AN ABSTRACT OF THE THESIS OF

Seong-Tshool Hong for the degree of **Master of Science** in **Biochemistry and Biophysics** presented on **March 15, 1993.**

Title : Choline-Lipid Release from Normal and Transformed Cells

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The effect of albumin on phosphatidylcholine (PC) metabolism in Hep-G2, 3T3-H.ras, and 3T3 cells prelabelled with [*Me*-^{3}H]choline was studied. The [^{3}H]choline was more rapidly taken up and more actively incorporated into cellular phospholipids in 3T3-H.ras cells than in Hep G2 and 3T3 cells. In each of the three cell lines, 97-98 % of the [^{3}H]choline metabolized into the phospholipids was incorporated into PC and 2-3 % into lysophosphatidylcholine (LPC). Bovine serum albumin stimulated the release of [^{3}H]LPC and [^{3}H]PC from each of the three cell lines pre-labelled with [^{3}H]choline. [^{3}H]PC was also released in the absence of albumin but [^{3}H]LPC was not. The efficiency of LPC secretion represented as the proportion of medium [^{3}H]LPC to cellular [^{3}H]choline lipid during a chase period is approximately 9 to 14 times greater in 3T3 cells

compared with the transformed 3T3-H.ras and Hep-G2 cells. A similar comparison of published data for rat hepatocytes with Hep G2 shows secretion to be 35-75 times greater from the rat hepatocytes. Also, PC secretion from 3T3 cells was 1.6 times more effective than from 3T3-H.ras, whereas rat hepatocytes secretes PC 2.8-3.8 times more effectively than does Hep G2. The measurement of specific radioactivity of cellular PC in pre-labelled 3T3 cells showed it to be similar to that of the secreted PC. However, the specific radioactivity of secreted LPC was markedly lower than that of the cellular PC, which suggests that LPC is being secreted from a PC pool distinct from that used for PC secretion.

Choline-Lipid Release from Normal and Transformed Cells

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I. INTRODUCTION

Phosphatidylcholine and lysophosphatidylcholine

Phosphatidylcholine (PC) is the major membrane phospholipid class in mammalian cells (1). It constitutes about one half of the phospholipids in eukaryotic cells and over half of serum phospholipids (1).

PC has a number of roles in the cell. In membranes the lipid plays a structural role and in serum lipoproteins it is present as a monolayer shell surrounding a core of nonpolar lipids (1). It serves as a substrate for lecithin:cholesterol acyltransferase in the plasma fraction of blood wherein it plays a role in cholesterol transport in the blood stream (2). In the form of dipalmitoyl PC, it is a critical component of pulmonary surfactant (2). In recent years, PC has attracted attention through the suggestion that it has a role as a substrate for the production of second messengers (3).

LPC generated from the hydrolysis of a fatty acid from PC also occurs naturally in mammalian tissues though at very much

lower levels than PC (1). However, it is a significant component of blood plasma and plays many physiological role.

Phosphatidylcholine biosynthesis

Free choline is brought into most non-neuronal cells by facilitated diffusion through a specific carrier-mediated transport mechanism (2). It is now well established that there are two distinct transport mechanisms for choline. One is a high-affinity (K_m < 5 μM) Na^+-dependent, and hemicholinium-3-sensitive mechanism tightly coupled to acetylcholine synthesis in cholinergic synaptosomes. The other is a low-affinity (K_m > 30 μM), relatively Na^+-independent and hemicholinium-3-insensitive mechanism found in most animal cells as well as in cholinergic nerve tissues. A situation in which the rate of choline transport is increased by increasing its substrate supply indicates that choline transport is not regulated. A nonsaturable component for choline transport has been detected in perfused rat liver (4), cultured rat hepatocyte (5), Ehrlich ascites cells (6), Novikoff hepatoma cells (7), and human erythrocytes (8). If choline transport is at equilibrium, then the effect of the increased extracellular choline is simply to increase the level of

intracellular choline, which then increases the rate of choline kinase.

The choline taken up is incorporated into PC via the CDP-choline pathway as shown in Figure 1 (9). Choline kinase catalyzes the first committed step, phosphorylation of choline to form phosphocholine. Phosphocholine and CTP are then converted to CDP-choline by CTP:phosphocholine cytidylyltransferase, and finally CDP-choline is incorporated into PC by choline phosphotransferase.

PC biosynthesis is mostly regulated by the enzyme CTP:phosphocholine cytidylyltransferase (9) which becomes active when it translocates from cytosol to endoplasmic reticulum membrane. There is evidence to suggest that free fatty acid stimulates this translocation. It has been reported that cellular PC biosynthesis is significantly elevated in 3T3 cells transformed with the Ha-ras oncogene through an increase in the cellular phosphocholine pool as a consequence of an activated choline kinase (14).

PC may also be synthesized by methylation of phosphatidylethanolamine with S-adenosylmethionine (1). Vance et al showed in rat hepatocytes that the PC pool synthesized from the CDP-choline pathway is preferred for PC secretion due to the

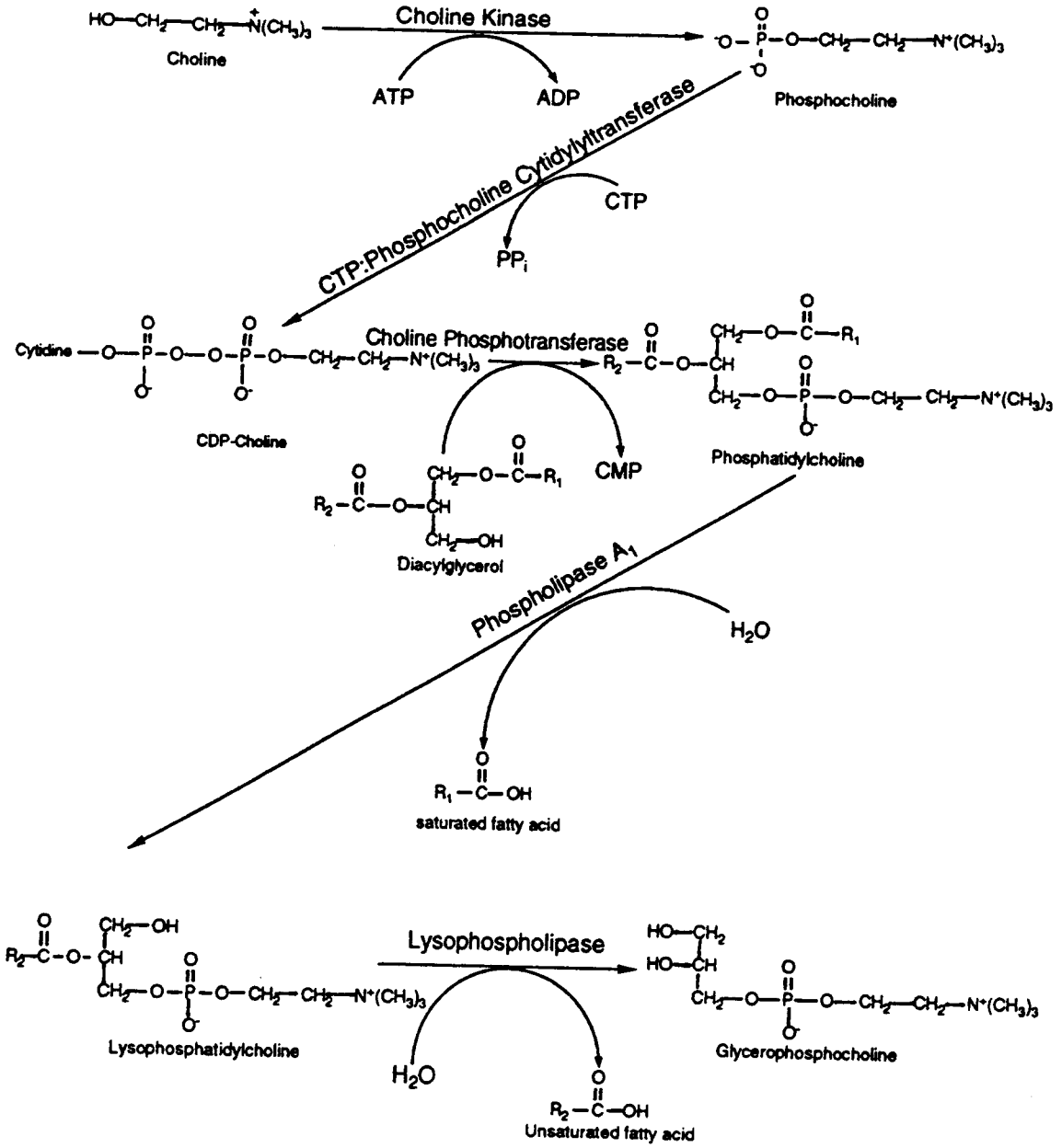

Figure 1. The PC synthesis and degradation pathway.

fact that the specific radioactivity of PC derived from [Me-^{3}H)choline was approximately equal in cells and medium (50). They also showed by measuring the medium and cellular specific radioactivity that although PC from the methylation of phosphatidylethanolamine, derived from ethanolamine, is not preferred for secretion, PC from the methylation of PE derived from serine is a preferred source of PC for secretion. This fact indicates that random and homogeneous PC pools are not selected for PC secretion. Similar conclusions were reported by Brindley et al (38). They observed a lower production of 20:4 fatty acid in cellular LPC from rat hepatocytes despite high production of this fatty acid in cellular PC. This suggests that a specific pool of PC molecular species is selected for PC turnover into LPC.

Phosphatidylcholine degradation

It has long been known that certain hormones, growth factors, and phorbol esters stimulate both PC synthesis (9-11) and PC hydrolysis (12,13) in 3T3 cell lines and other cells. These factors thereby increase the PC turnover rate.

Since PC degradation can lead to the release of arachidonic acid and diacylglycerol, the PC turnover cycle is recognized as a

potential source of these second messengers during signal transduction by hormones, i.e. it may serve as a means of long term maintenance of the level of diacylglycerol produced by the well known signal transduction pathway via the hydrolysis of phosphatidylinositol-4,5-bisphosphate (3,15). In this context, G-proteins are implicated in the coupling of agonist receptors to the activation of phospholipases A_1, A_2, C, and D. Figure 2 outlines this turnover cycle in mammalian cells.

Membrane PC and LPC exist in a dynamic flux in which continuous biosynthesis is balanced by degradation (9). Experiments using isolated organs, tissue slices and homogenates have shown that the half-lives of cellular PC vary among different tissues from 1.2 hour in rat liver to 1350 hours in rat brain cells (16). The considerable variation of half-lives was found to be independent of the precursor used. This indicates that the rate of cellular LPC production from PC will vary among different tissues. Interestingly, studies with cells in tissue culture have shown that PC turnover was similar for growing and nongrowing cells under conditions where no secretion of phospholipids into the medium took place (16).

Lysophosphatidylcholine metabolism

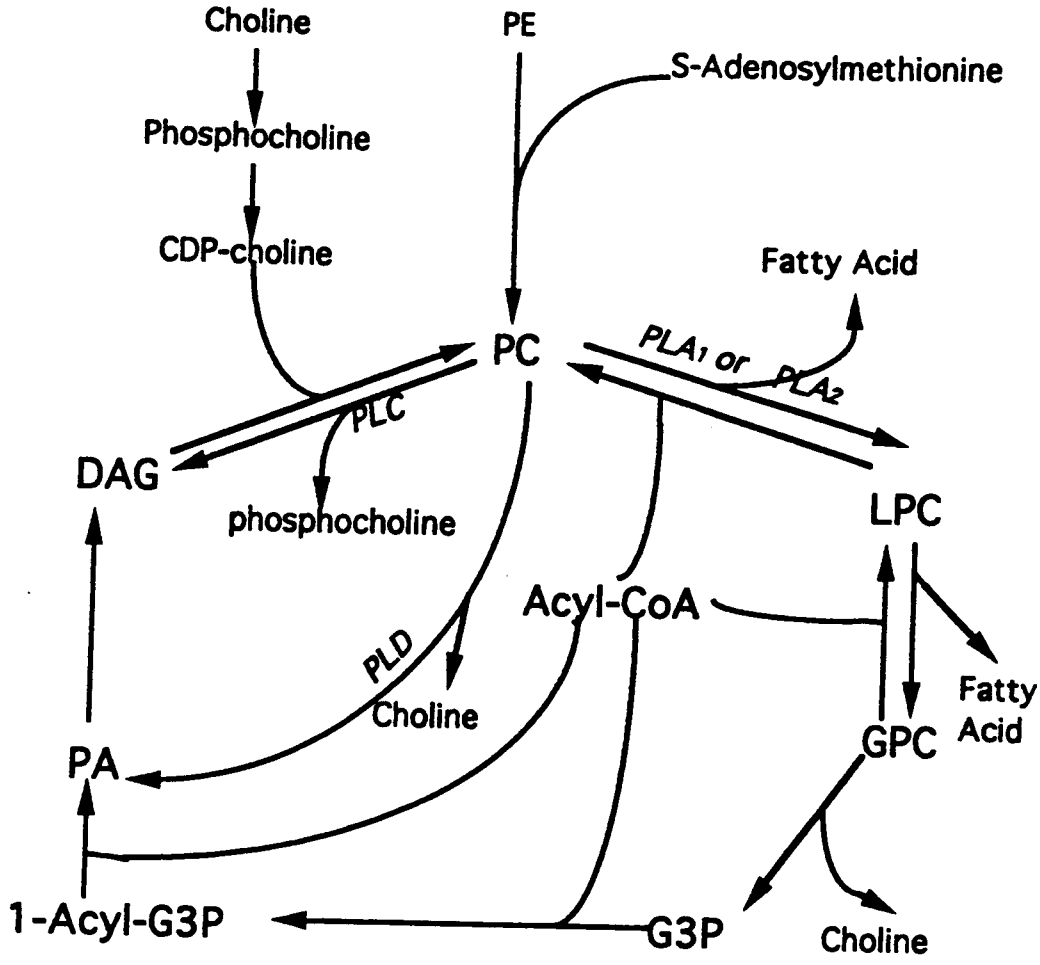

Figure 2. PC turnover cycles. The DAG, PC, LPC, GPC, G3P, PE, and PA means diacylglycerols, phosphatidylcholine, lysophosphatidylcholine, glycerophosphatidylcholine, glycerol-3-phosphate, phosphatidylethanolamine, and phosphatidic acid respectively. The PLA_1, PLA_2, PLC, and PLD represent phospholipase A_1, A_2, C, and D.

LPC generated during PC metabolism may be converted to PC through reacylation or to glycerophosphocholine (GPC) by hydrolysis of the remaining fatty acid (figure 2). Two enzymes are responsible for the acylation of LPC (2). Lysolecithin acyltransferase transfers a fatty acyl-CoA to the 2-position of 1-acyl-LPC whereas 2-acylglycerophosphocholine acyltransferase transfers a fatty acyl-CoA to the 1-position of 2-acyl-LPC. These reactions have been shown to be very important for the synthesis of PC in liver.

However, a quite different type of acylation of LPC was discovered by Marinetti (17). The reaction requires two molecules of 1-acyl-LPC and yields PC and GPC. This pathway was considered of only minor importance in liver but a major pathway in lung.

LPC can be further degraded by lysophospholipase which converts it to a fatty acid and GPC. The GPC is known to be subsequently hydrolyzed to choline and glycerol phosphate, or glycerol and phosphocholine by a phosphodiesterase (2).

Physiological role of lysophosphatidylcholine

At present it is unknown whether LPC, generated in the PC

turnover cycle, may play any role in signal transduction. However, LPC seems to play important physiological roles either in the form of cellular LPC or as plasma LPC. Cellular LPC constitutes less then 2% of the total phospholipids in the membranes of most cells (1) but it is present in significant amounts in plasma (18). The circulating plasma LPC exerts several important physiological effects. It affects platelet aggregation (19), erythrocyte agglutination(20), and cardiac arrhythmia (21). It also acts as the binder of low density lipoprotein to arterial cells (22) and as a chemotactic factor for human monocytes (23). A decrease in protein-mediated glucose transport in red blood cells also occurs with physiological concentrations of LPC (24). The demyelination of various mammalian nerve tissues by LPC has also been observed (25-29).

Plasma LPC are mostly produced in the plasma by the action of lecithin-cholesterol acyltransferase (30), which catalyzes the transfer of a fatty acid from the 2-position of PC to cholesterol. In this case LPC is a coproduct with the formation of cholesterol esters. Since LPC is readily taken up by cells, the circulating plasma LPC may be regarded as a source of fatty acid and choline by extravascular tissues (31). However, plasma LPC has also been shown to originate directly from liver in addition to

being generated by plasma lecithin-cholesterol acyltransferase (32). It has been reported that albumin stimulates the release of LPC from isolated perfused rat liver (32) and monolayer cultures of rat hepatocyte (34-38). The released LPC from the liver and hepatocyte appear to be formed by the action of phospholipase A_1 or A_2 on cellular PC. This hepatic LPC is predominantly unsaturated, which is markedly dissimilar from the highly saturated LPC synthesized by plasma lecithin-cholesterol acyltransferase activity in plasma. In the previous work (34-38), it was proposed that LPC secreted from liver is a major source of plasma lysophosphatidylcholine due to the fact that LPC concentration in the plasma remains relatively high even in patients suffering from a deficiency of lecithin-cholesterol acyltransferase (39).

Choline-Lipid research On Hep-G2, 3T3, and 3T3-H.ras cells

Cell culture systems of eukaryotic tissues have provided many insights into biochemical pathways and mechanisms. Metabolic studies of tissues and tissue-derived tumors have been greatly facilitated by the isolation of cell cultures. The manipulation of culture conditions has permitted studies of

nutrient requirements and the role of hormones and other factors on metabolic pathways. In addition, comparison of the biochemistry of a specific cell type and its transformant is made much simpler. The fact that many cell lines are immortal allows one to dispense with the sacrificing of laboratory animals to carry out metabolic studies.

Hep-G2, a liver cell line derived from a human hepatoma that is free of known hepatotropic viral agents, has been found to express a wide variety of liver-specific metabolic functions including cholesterol and triglyceride metabolism (40). The lipid composition of Hep G2 cells is close to that of normal human liver, except for a higher content of sphingomyelin and a lower PC/sphingomyelin ratio (41). Studies on the effects of fatty acids on PC synthesis in Hep-G2 have shown that CTP:phosphocholine cytidylyltransferase activity was increased by free fatty acid only in membrane fractions (42) as in the case of mammalian hepatocytes.

3T3 cells, undifferentiated fibroblasts, have served as a good model for the action of hormones and growth factors on peripheral tissues. Thus, it is not surprising that most recent papers on PC metabolism in 3T3 cells deal with the signal transduction through PC hydrolysis either by phospholipase C or

by phospholipase D. Warden and Friedkin have reported that treatment of Swiss Balb c 3T3 mouse embryo fibroblast with fetal bovine serum increased the intracellular choline kinase activity by 2-3 fold. This led to an increased pool size of phosphocholine and enhanced PC biosynthesis (10). The PC hydrolysis in second messenger generation in 3T3 cells has been demonstrated by the finding that phospholipase D-mediated hydrolysis of PC can be activated by various growth factors and hormones (44). 1,2-Diacylglycerol, produced from phospholipase C and also as a product of phospholipase D in combination with phosphatidate phosphatase, is known to stimulate protein kinase C. Chatopadhyay et al. showed that the activated protein kinase C stimulates PC synthesis but the increased PC hydrolysis by protein kinase C activators is not necessarily associated with increased PC synthesis (45). However, there is evidence for a PC-specific phospholipase C, which may be responsible for the activation of PC turnover in 3T3 cells (46,47) and also DNA synthesis activation (47).

In 3T3 cells transformed by the H.ras oncogene, increased diacylglycerol levels have been found, which most probably arise from activation of the turnover of PC (48). Interestingly, the elevated cellular phosphocholine pool via increased choline

kinase activity was also observed in H.ras-transformed 3T3 cells (14). Diacylglycerol is a key activator of protein kinase C, whose role in cell growth and transformation has been proposed. It is known that the protein kinase C translocates from the cytosol to the plasma membrane during stimulation of the enzyme. Diaz-Laviada et al showed by using immunochemical techniques that transformation by H.ras oncogene is associated with permanent translocation of protein kinase C to the cytoplasmic membrane to activate permanently the enzyme without down regulation (49).

Purpose of this research

Based on the observation that albumin stimulates the release of LPC from monolayer cultures of rat hepatocytes, we first examined whether this albumin-stimulated secretion of LPC is unique to hepatocytes or is a phenomenon which might be displayed by undifferentiated and also transformed cells which have active lipid metabolism. For this purpose, we used the three cell lines, 3T3, 3T3-H.ras, and Hep G2.

PC is a prominent component of all plasma lipoproteins and made both by the CDP-choline pathway and by methylation of

phosphatidylethanolamine in the liver (1). Since Vance et al had already shown that the PC secreted by hepatocytes originates from a selected cellular PC pool, the second objective of this research was to compare the specific radioactivities of cellular and secreted PC and LPC pools generated from [^{3}H-Me]choline. Such a comparison might enable us to establish whether the secreted LPC has an origin different from that selected for PC secretion.

In previous studies (31,35-38), it was proposed that the release of LPC from rat hepatocytes stimulated by albumin results from the ability of albumin to bind to cellular LPC located in the plasma membrane. Cancer cells show different and decreased amounts of glycoprotein and glycolipids in the cell membranes compared with host cells (51,57). Differences are also observed in membrane-bound proteolytic enzymes and the cell skeleton (51). However, tests so far show that the phospholipids are unchanged in cancer-transformed cells (51). Comparisons of the rates of LPC secretion between 3T3 and 3T3-H.ras cells and of the previously studied rat hepatocytes with Hep G2 might reveal differences which correlate with the difference in their plasma membrane structures resulting from the transformations.

II. MATERIALS AND METHODS

Materials.

Fetal-calf serum and powdered formulations of Ham's F-12 nutrient mixture (HAMS), high-glucose Dulbecco's modified Eagles medium (MEM), and choline and methionine-free Eagle's minimum (MEM-) medium were obtained from Gibco Laboratories (Island, New York, USA). [Me-^{3}H]choline chloride (specific radioactivity 76 Ci/mmol; radiochemical purity 98 %) was obtained from Amersham Corporation (Arlington Heights, IL, USA). Coomassie brilliant blue G-250 reagent was from Bio-Rad Laboratories, Richmond, CA, USA. Silica gel G tlc (20x20 cm) tlc plates were from Alltech Associates, Inc., 2051 Waukegan Road, Deerfield, IL 60015, USA. Fatty acid-free albumin, trypsin, and all other chemicals were reagent grade and obtained from Sigma Chemical Co., St. Louis, MO, USA. Cell culture Falcon dishes (60mmX15mm) were obtained from Fisher Scientific Co., Pittsburg, PA, U.S.A..

Cell lines.

All the cell lines used in this study were kindly provided by Dr. David Barnes, Department of Biochemistry and Biophysics, Oregon State University. The human hepatocellular carcinoma cell line Hep G2 and mouse embryo fibroblast BALBc/3T3 clone A31 cell line (3T3) were from the American Type Culture Collection (ATCC) cell bank. The 3T3-H.ras line was prepared in David Barnes' laboratory from plasmid pUC EJ6.6 which contained the activated H.ras gene under control of the endogenous promotor.

Incubation of cells.

The three cell lines , 3T3 (mouse embryo fibroblast cell), 3T3-H.ras and Hep-G2, kept in 10% dimethyl sulfoxide at -70 °C were brought to -30 °C for a half hour and then room temperature for a half hour. The cells relieved from frozen condition were washed and suspended in Eagle's minimum medium. The cells were collected by low speed centrifugation and the medium containing dimethyl sulfoxide discarded. The collected cells were resuspended in Dulbecco's modified Eagle's medium HAMS:MEM

(1:1), pH 7.4, in a 250-mL Falcon bottle, containing 1.7 μM insulin, and 10% fetal calf serum for the Hep G2 and 10% calf serum in HAMS:MEM (1:9), pH 7.4, for the 3T3 and 3T3-H.ras. The cells were incubated at 37 °C under an atmosphere of air/CO_2(19:1) until complete coverage of cells on the bottom of the bottle. The medium was replaced when necessary, usually every two to three days. After complete cell coverage of the bottom of the bottle, the growth medium was removed and 1 ml of 0.2 % trypsin (w/v) in PBS, which was 1 mM in EDTA, was added. After a few minutes the cells were freed and the trypsin reaction was stopped by adding 10 ml of Eagle's minimum medium containing the calf serum. The cell suspension was transferred into 50 ml-plastic centrifuge tubes and centrifuged for 5 minutes at 600 rpm to collect the cells. The medium containing trypsin was removed and the cells were resuspended in 25 ml of choline and methionine free Eagle's minimum medium. Cell number was determined by Coulter counter. Equal numbers (1 to $1.5x10^6$) of cells were dispersed into plastic culture dishes in 2 ml of the medium to give a subconfluent layer. Before labelling, the cells were maintained overnight in the choline- and methionine-free medium to deplete the cellular choline and phosphocholine pools.

Incorporation of [*Me*-^{3}H]choline.

The dishes of cultured cells were washed two times with 2 ml of serum-free minimum medium to remove non-viable cells. Each dish of cultured cells was incubated with 2 ml of choline-free medium that contained 10 µCi of [*Me*-^{3}H]choline chloride (76ci/mmol). After 1 hour the cells were washed twice with the chase medium, which was composed of MEM:HAMS (1:1) containing unlabelled 28 µM choline. The labeled cells were subsequently incubated for up to 4 hr with 2 ml of the chase medium. The chase medium was either albumin-free as a control or contained 5 mg/ml bovine serum albumin for the experimental runs. At the end of the incubation period, the medium was removed and the monolayer of cells washed with 1 ml of phosphate buffered saline. The medium and cell washings were combined, and centrifuged at 10000 g for 20 min to remove detached cells and cell debris. The supernatant was used for extraction and analysis of separated lipids. The cells were scraped from the dishes in 2 ml of distilled water with a rubber policeman. The cell suspension was sonicated for 30s and, after removal of 50 µl for protein analysis, was used for extraction and analysis of lipids.

Extraction and analyses of cell and medium lipid.

Lipid was extracted from sonicated cell preparations and centrifuged media by adding 3 volume of chloroform/ methanol(2:1, v/v) containing the antioxidant 2,6-di-t-butyl-4-methylphenol (50 mg/l) using the method of Folch et al. (24). After centrifugation of the extract at 300 g for 1 hour, the upper aqueous phase was removed and the lower chloroform phase washed once with 4 ml of methanol/water (1:1, v/v). A portion of the lower chloroform phase (0.1ml) containing lipids was evaporated to dryness under N_2 in a scintillation vial. The residue was dissolved in 4 ml of scintillation fluid (0.4 %, w/v, of PPO in toluene: Triton X-100: H_2O, 6:3:1, v/v/v) and radioactivity was measured with a Beckman liquid scintillation counter (LS-230). The remainder of the lower chloroform phase containing phospholipids was evaporated to small volume and applied to a silica-gel 60 t.l.c. plate, which was developed in the solvent system chloroform/methanol/acetic acid/water (50:30:8:3 by vol.) for the separation of phospholipids. After the plate was air-dried, the radioactivity spots of PC and LPC were observed with I_2 vapor and by radioactivity scanning of the plate using a Bioscanner (400 imaging scanner) . The labeled PC and LPC zones were scraped

from the tlc plate and transferred to Pasteur pipettes that were plugged with a small cotton plug to retain the scrapings. The lipids were eluted from the SiO_2 scrapings with 15 ml chloroform/methanol (2:1). The eluate was evaporated to a volume of 2 ml and an aliquot (0.1 ml) was used for radioactivity determination. The remaining 1.9 ml was used for phosphate assay.

Phosphate assay.

Phosphate content of phospholipids was measured by the modified Fiske and Subbarow method(52). The phospholipid samples isolated in chloroform and methanol mixture were dried by using N_2 gas. Concentrated perchloric acid (450 µl) was added to the dried sample and heated in a sand bath at 180 °C. After 1 hour, 2.5 ml distilled water, 0.5 ml 10 % ascorbic acid, and 0.5 ml 2.5 % ammonium molybdate were added and vortexed in order. The mixture was heated at 100 °C for 15 minute. After cooling to room temperature, the absorbance at 820 nm was measured. Inorganic phosphate was used as the standard.

Protein determination.

The amount of cellular protein was determined using the Coomassie brilliant blue G-250 from Bio-rad Laboratories. The sonicated cell suspension, 50μL, was dissolved in 0.1 M NaOH, 0.8 ml, and then incubated at 55°C for 10 minute. After addition of 0.2ml of the Coomassie reagent, the optical density was measured at 595 nm. Typically, the protein content of the cells in a culture dish was 280 to 380 μg/mL.

III. RESULTS

Incorporation of (^{3}H)-choline into cellular phospholipid.

After preincubation of cells with [*Me*-^{3}H]choline, continued incubation in the presence of cold choline (the chase period) allowed the cells to metabolize and secrete the newly generated lipids. The extracted lipids from the cells and the incubation media were separated by tlc. A typical scan of the radioactivity of the cellular lipids is shown in Figure 3. The ^{3}H is located exclusively in LPC and PC with ca. 97 % being associated with PC.

After the initial 1 hour pulse with the labelled substrate, the percent absorbed by 3T3, 3T3-H.ras, and the Hep-G2 cell lines was 32.8±0.6, 65.3±0.4, and 51.3±1.3 % respectively. The time-dependent changes in radioactivity associated with cellular and secreted metabolites are expressed in terms of specific activity (dpm/mg cellular protein) so as to normalize the data for the small variation in cell number per dish.

The presence of albumin in the incubation medium during the chase period has no marked effect on the radioactivity in the

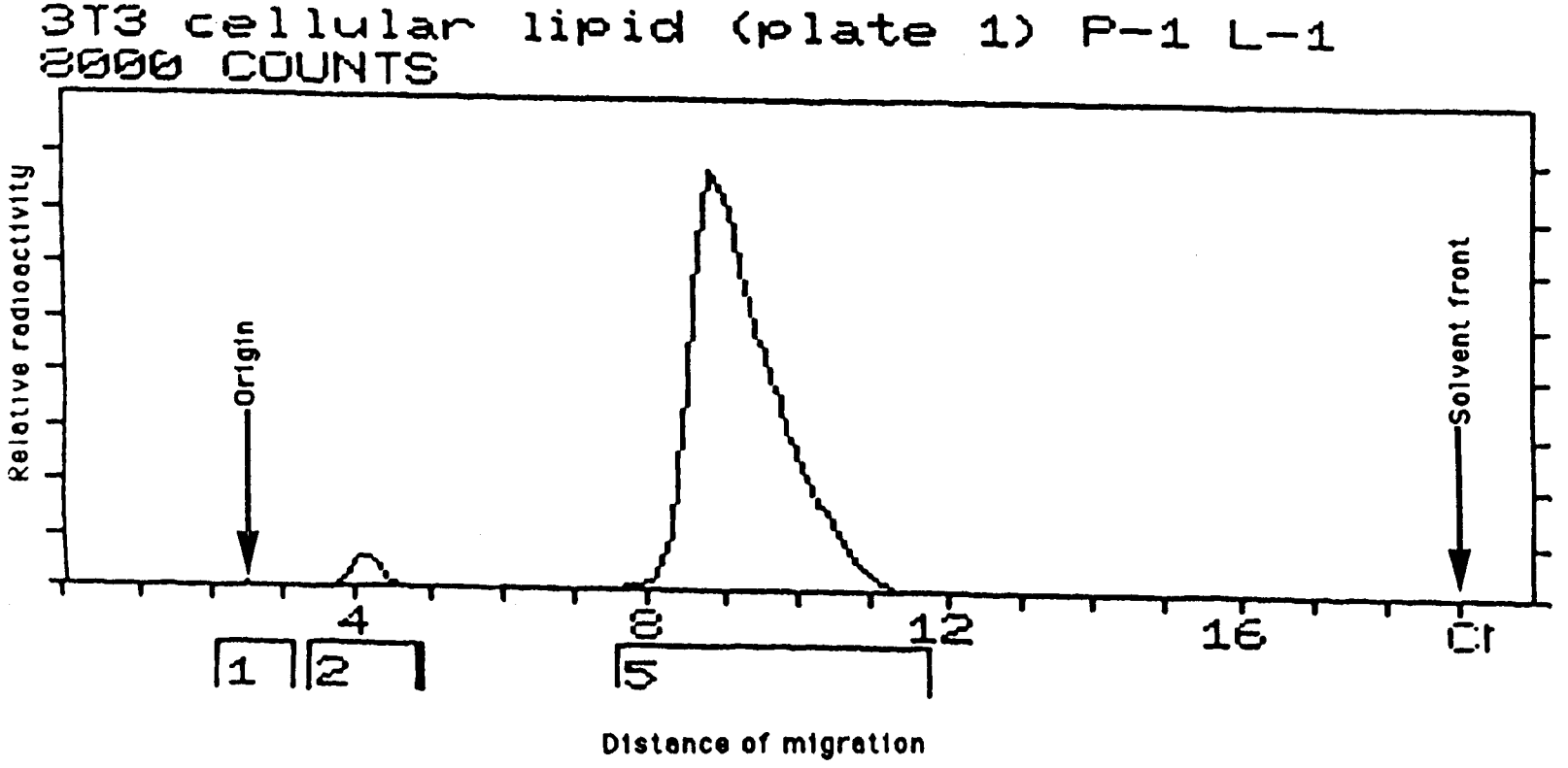

Figure 3. Radioactivity distribution of cellular [^{3}H]lipid compounds of 3T3 cells on tlc detected by automatic radioactivity Bioscanner. The cells prelabeled with [^{3}H]choline were collected for lipid analysis after further 1 hour chase with albumin. This radioactivity distribution pattern was similar in all three cell lines regardless of albumin addition. Zones [2] and [5] represent LPC and PC respectively.

total cellular lipid or in either cellular PC or LPC in any of the cell lines as shown in Figures 4, 5, and 6. Whereas Hep G2 and 3T3 cells continue to accumulate [^{3}H]choline in their lipids during the four hour chase, PC labelling in 3T3-H.ras cells is close to a plateau level during the chase.

As can be seen in Figure 3, most of the radioactivity (about 97%) was incorporated into PC and about 3% into LPC in each of the three cell lines. So, it is not surprising then that the radioactivity incorporation patterns of total cellular [^{3}H]lipid from [^{3}H]choline in Figure 4 reflects the radioactivity profiles of cellular [^{3}H]PC in Figure 5. Cellular [^{3}H]LPC during the chase period (figure 6) shows some small accumulation but the presence of albumin has no significant effect in any of the three cell lines.

Release of lysophosphatidylcholine and phosphatidylcholine by albumin as a function of time

The most conspicuous result in this study was the difference in release of LPC into the albumin-containing medium from cultured 3T3, 3T3-H.ras, and Hep-G2 cells during the time course. In this experiment bovine serum albumin, free of lipid and other small metabolites, was used to promote LPC secretion.

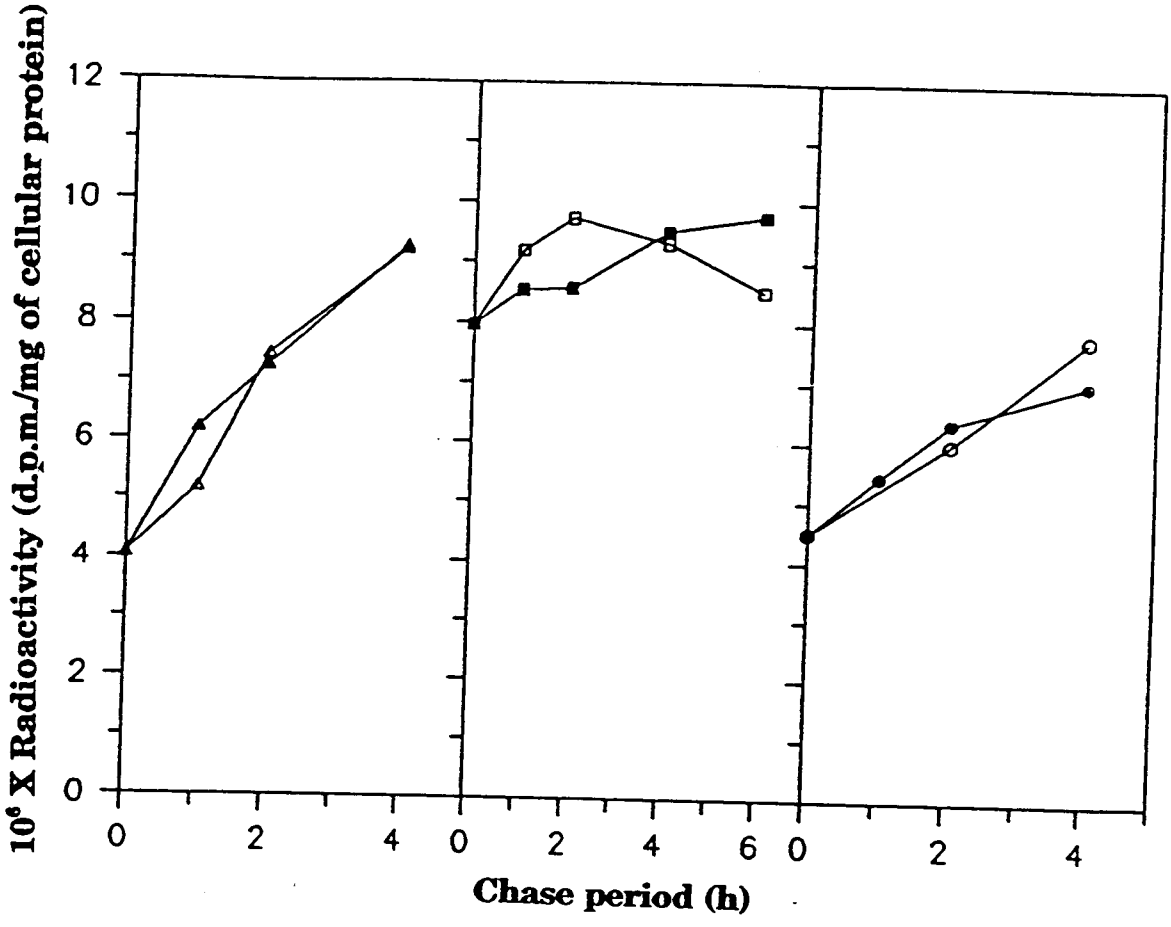

Figure 4. Effect of albumin on the metabolism of cellular [^{3}H]lipids in three cell lines prelabelled with [*Me*-^{3}H]choline. The cells were collected at the times indicated and radioactivity was determined as described in the experimental section. Each point represents the mean of two or three dishes, and the ranges were less than 10 % of the mean. Closed symbols are for albumin-containing incubation media and open symbols are for albumin-free media. ▲,□,○ represent Hep G2, 3T3-H.ras, and 3T3 respectively.

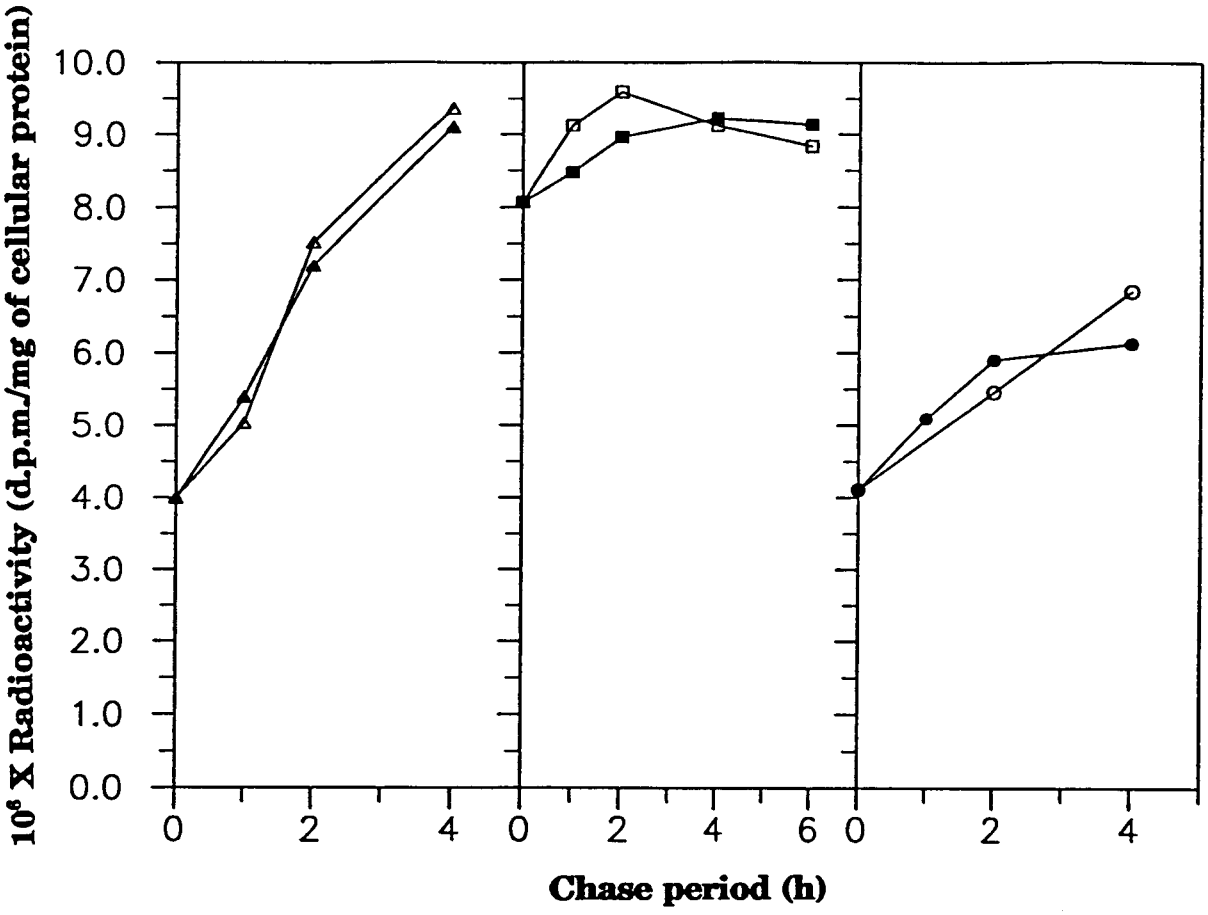

Figure 5. Effect of albumin on the cellular [^{3}H]PC radioactivity changes in three cell lines prelabelled with [*Me*-^{3}H]choline. The cells were collected at the times indicated and the radioactivity was determined as described in the experimental section. Each point represents the mean of two or three dishes, and the ranges were less than 10 % of the mean. Closed symbols are for albumin-containing incubation media and open symbols are for albumin-free media. △,□,○ represent Hep G2, 3T3-H.ras, and 3T3 respectively.

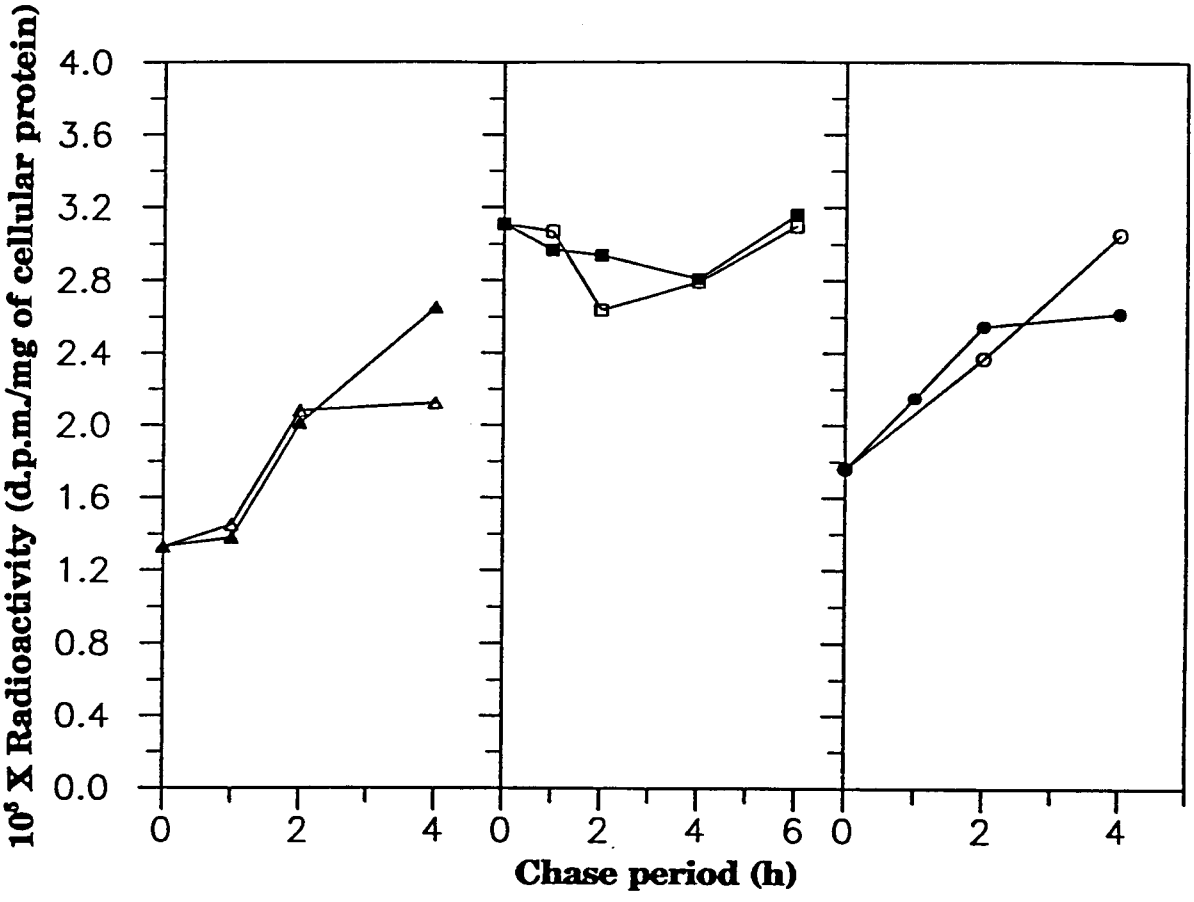

Figure 6. Effect of albumin on the cellular [^{3}H]LPC radioactivity changes in three cell lines prelabelled with [*Me*-^{3}H] choline. The cells were collected at the times indicated. Radioactivity was determined as described in the experimental section. Each point represents the mean of two or three dishes, and the ranges were less than 10 % of the mean. Closed symbols are for albumin-containing incubation media and open symbols are for albumin-free media. ▵,□,○ represent Hep G2, 3T3-H.ras, and 3T3 respectively.

Figures 7 and 8 show the radioactive scans of the separated lipids secreted from pre-labelled 3T3 cells during a 1 hour chase into an albumin-containing and an albumin-free medium respectively. It is clear that with albumin present the [^{3}H]LPC is released along with [^{3}H]PC whereas only [^{3}H]PC appears in the absence of albumin. Figure 9 shows the influence of albumin on the release of total [^{3}H]choline lipids into the media of each of the three cell lines during the four hour chase. Again it is clear that albumin has a stimulating effect upon this release of lipid with the effect being most marked for 3T3 cells. A time course of the albumin-mediated release of [^{3}H]LPC from each of the three cell lines is presented in Figure 10. The most striking observations are that in the absence of albumin no [^{3}H]LPC is released into the medium from any of the three cell lines and that release of [^{3}H]LPC from the 3T3 cells is much greater than that from the transformed cell lines.

Clearly, with albumin present, all three cell lines release [^{3}H]LPC but they do so with markedly different efficiencies. Tables 1 and 2 compare these efficiencies by expressing them as ratios of the [^{3}H]LPC released during a 1 hour interval divided by the [^{3}H]choline lipid present in the cell at the beginning of the interval. Table 1 shows that 3T3 secretes [^{3}H]LPC 9-14 times more

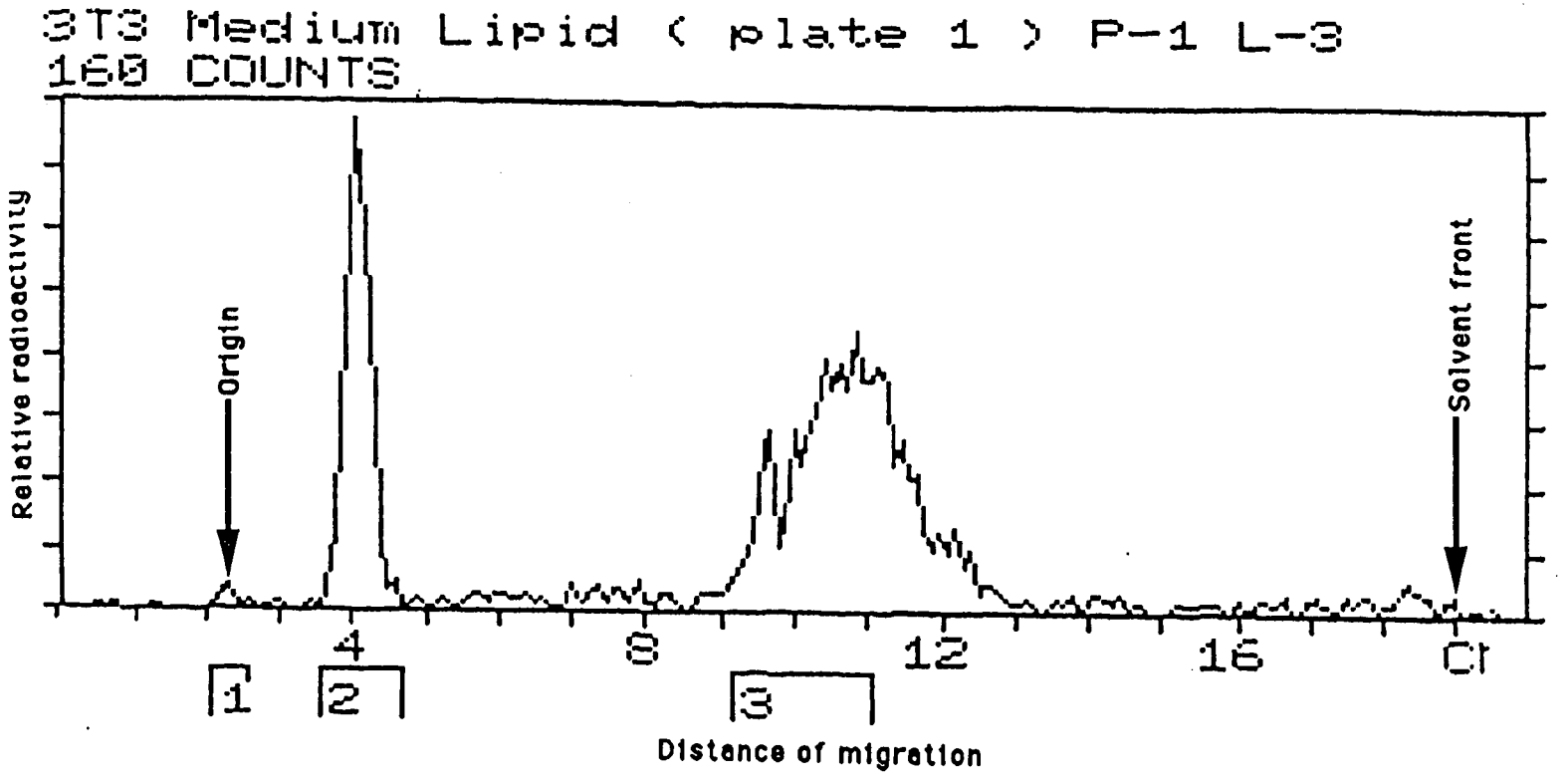

Figure 7. Radioactivity distribution of [^{3}H]lipid secreted from 3T3 cells into an albumin containing medium. The medium lipid secreted from 3T3 cells prelabeled with [^{3}H]choline were collected for analysis after a 1 hour chase. Zones [2] and [3] represent LPC and PC respectively.

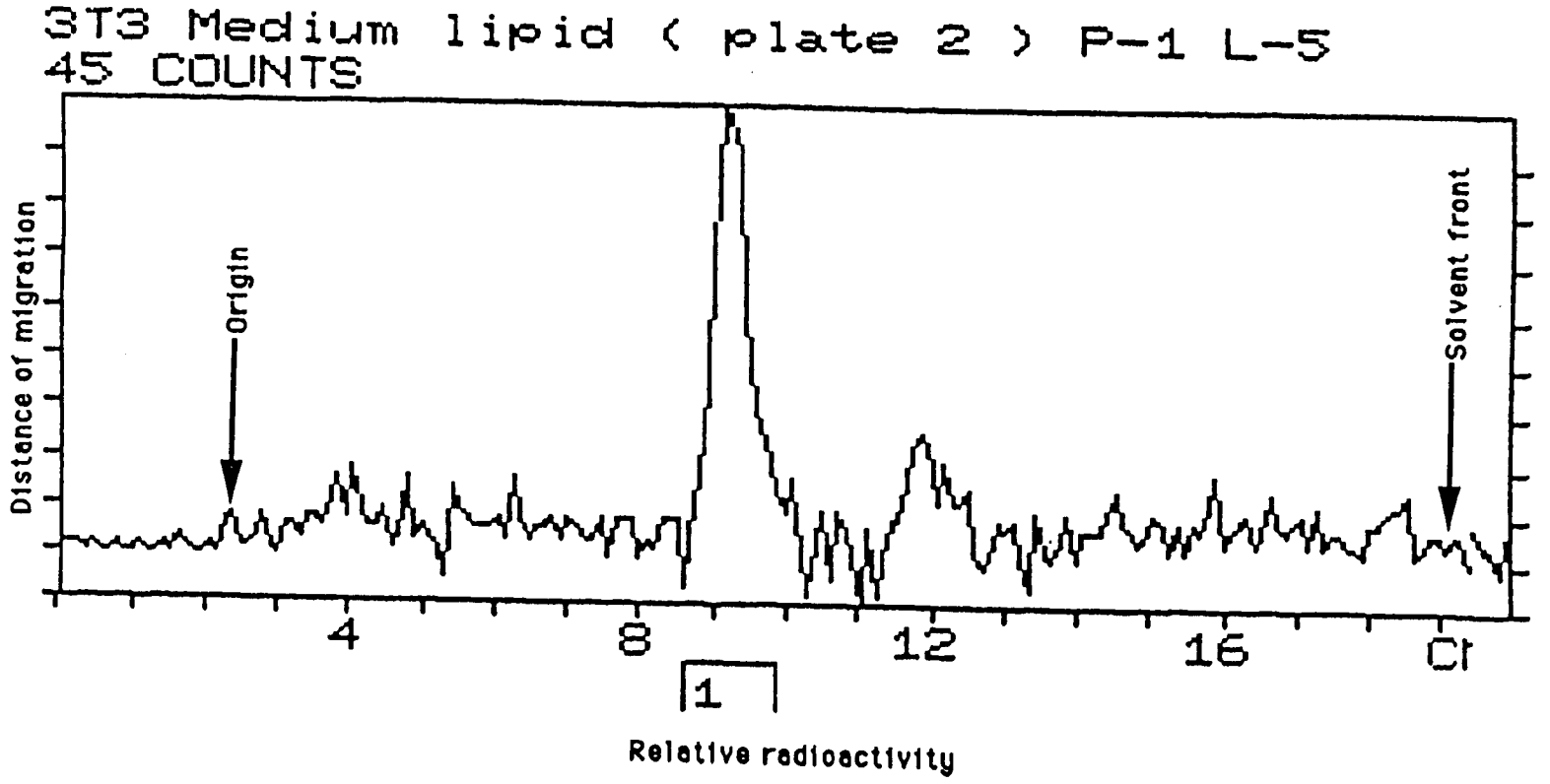

Figure 8. Radioactivity distribution of [^{3}H]lipid secreted from 3T3 cells into an albumin-free medium. The medium lipid secreted from 3T3 cells prelabeled with [^{3}H]choline were collected for analysis after a 1 hour chase. Zone [1] represents PC.

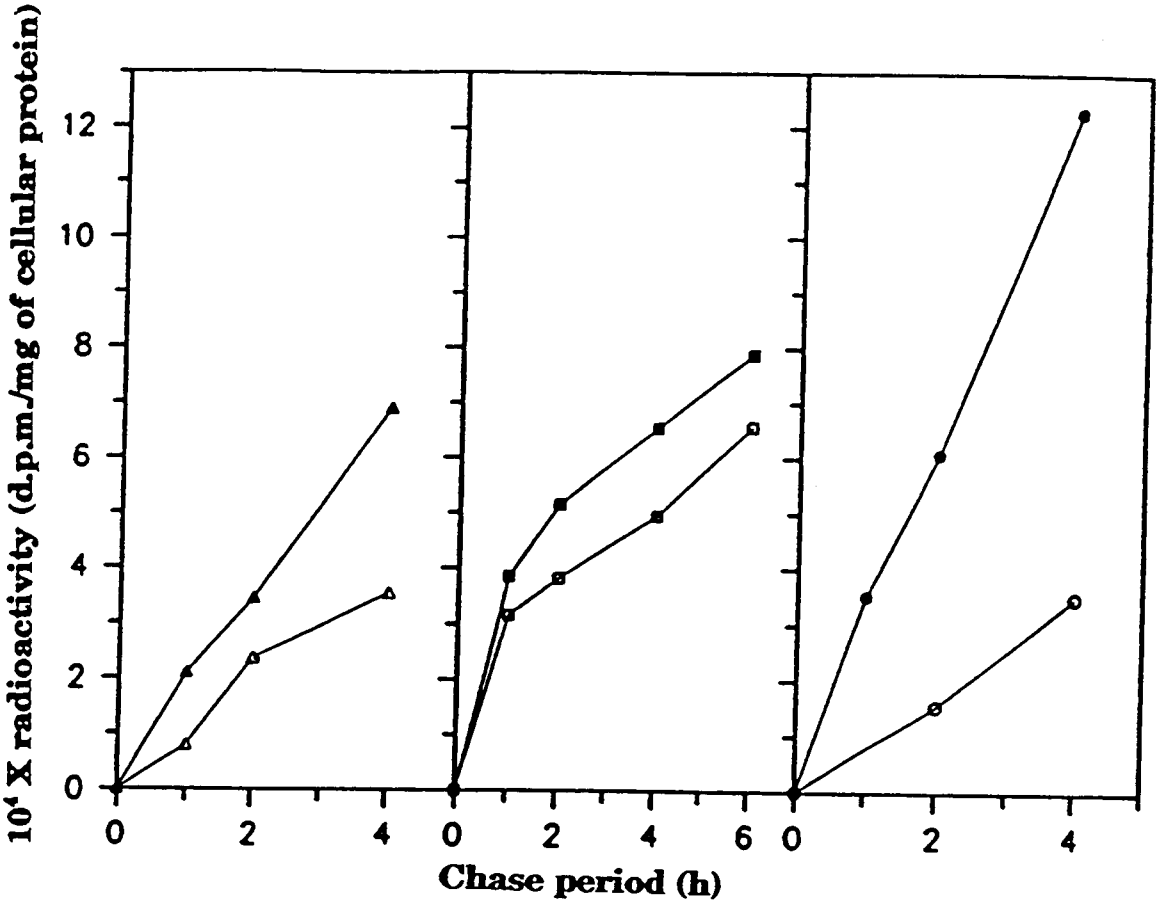

Figure 9. Effect of albumin on the release of [^{3}H]lipids from three cell lines prelabelled with [*Me*-^{3}H]choline. The medium was collected at the times indicated and radioactivity was determined as described in the experimental section. Each point represents the mean of two or three dishes, and the ranges were less than 10 % of the mean. Closed symbols are for albumin-containing incubation media and open symbols are for albumin-free media. ▲,■,● represent Hep G2, 3T3-H.ras, and 3T3 respectively.

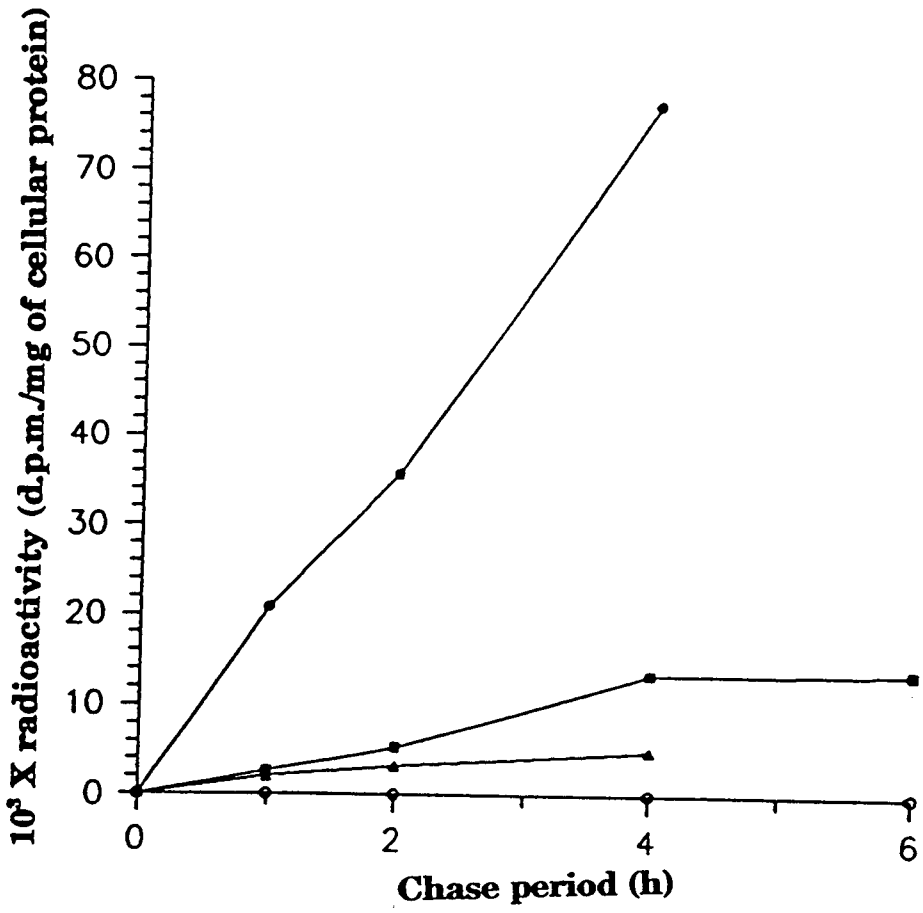

Figure 10. Effect of albumin on the release of [^{3}H]LPC from three cell lines prelabelled with [*Me*-^{3}H]choline. The medium was collected at the times indicated and radioactivity was determined as described in the experimental section. Each point represents the mean of two or three dishes, and the ranges were less than 10 % of the mean. Closed symbols are for albumin-containing incubation media and open symbols are for albumin-free media. ▵,□,○ represent Hep G2, 3T3-H.ras, and 3T3 respectively.

Table 1. Comparison of the efficiency of release of [^{3}H]-LPC from 3T3-H.ras with that from 3T3. [^{3}H]-LPC was isolated from the medium of pre-labelled cells at the indicated times as described in the Materials and Methods section. The radioactivity ratios represent the released labelled LPC during a 1 hour chase interval divided by the amount of labelled cellular choline-lipids present at the begining of the interval. Each value of the ratios is the mean ± S.D. of two or three analyses. C.C.-lipid means total cellular choline lipid

Incubation Time	medium LPC/C.C.-lipid in 3T3-H.ras (%)	medium LPC/C.C.-lipid in 3T3 (%)
1	0.033±0.001	0.462±0.019
2	0.031±0.007	0.273±0.007

Table 2. Comparison of the efficiency of release [^{3}H]-LPC from Hep G2 with that from normal rat hepatocytes. For the Hep-G2, [^{3}H]-LPC was isolated from the medium of pre-labelled cells at the indicated times as described in the Materials and Methods section. The radioactivity ratio represent the released labelled LPC during a 1 hour chase interval divided by the amount of labelled cellular choline-lipids present at the begining of the interval. Each value of the ratios is the mean ± S.D. of two or three analyses. C.C.-lipid means total cellular choline-lipid.

Incubation Time	medium LPC/C.C.-lipid in Hep G2 (%)	medium LPC/C.C.-lipid+ in rat hepatocytes (%)
1	0.049±0.000	1.71±0.17
2	0.019±0.009	1.43±0.14

+, calculated from previously published data obtained under the same experimental conditions (35).

effectively than that from 3T3-H.ras. In Table 2, the results for Hep G2 are compared with those for rat hepatocytes calculated from previously published data obtained under the same conditions as described in this research. Rat hepatocytes secrete LPC 35-75 times more effectively than do Hep-G2 cells. It is obvious that Hep G2 is the least efficient in [^{3}H]LPC secretion among these cells while rat hepatocytes are the most efficient.

Albumin also stimulates PC secretion into the chase medium as well as LPC. The effect of albumin on the release of [^{3}H]PC from cultured 3T3, 3T3-H.ras, and Hep G2 prelabelled with [^{3}H]choline is depicted in Figure 11. It is clear that PC is secreted into the medium in each of the three cell lines in the absence of albumin. However, Hep G2 secretes PC most effectively of the three cell lines. It is noteworthy that albumin is more stimulatory on the 3T3 and 3T3-H.ras cells than Hep G2 cells. At 4 hours of chase PC secretion by albumin from Hep G2 is increased about 25 % whereas the increase is about 60 % and 40 % respectively for 3T3-H.ras and 3T3 cells. Even so, the liver cell line is much more efficient at [^{3}H]PC secretion than the two fibroblast lines with or without albumin present. Tables 3 and 4 show that the transformed cells release [^{3}H]PC less effectively than non-transformed cells in the presence of albumin. Rat hepatocytes

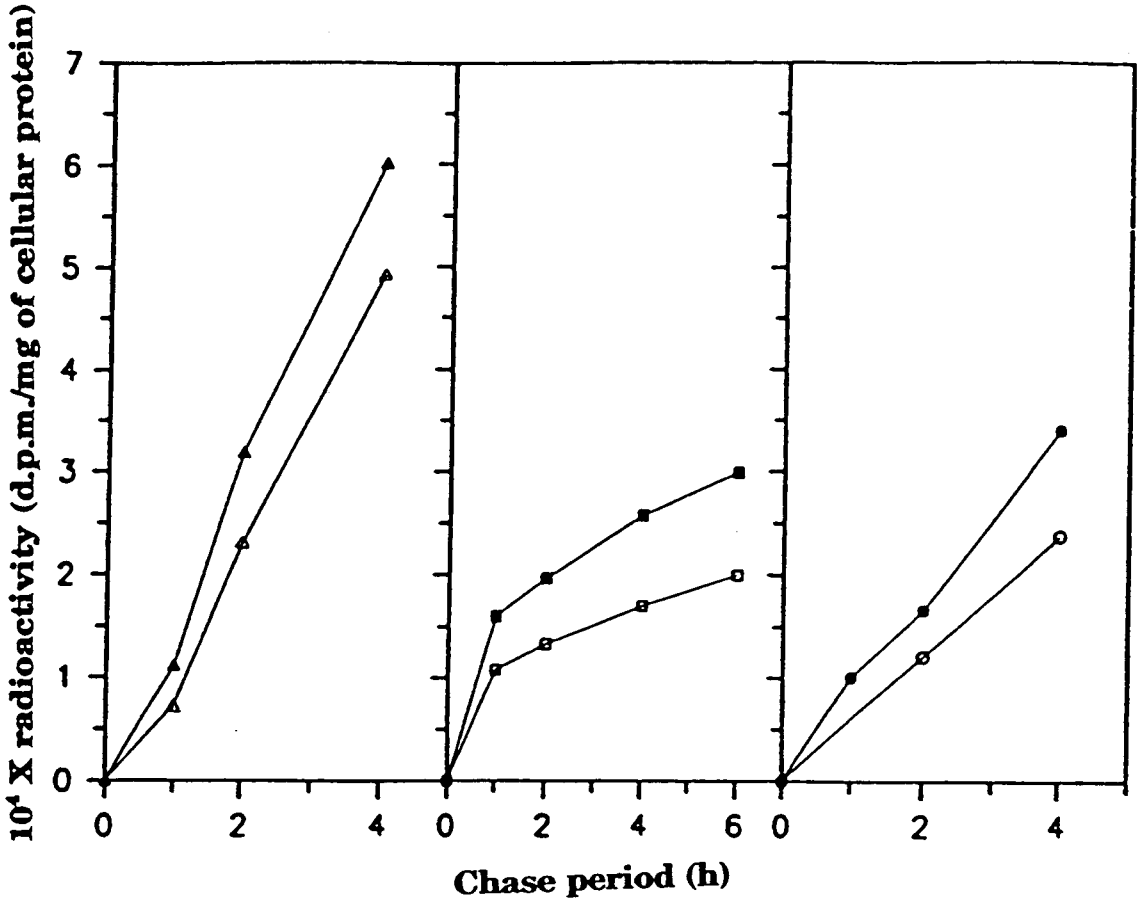

Figure 11. Effect of albumin on the release of [^{3}H]PC from three cell lines prelabelled with [*Me*-^{3}H]choline. The medium was collected at the times indicated and radioactivity was determined as described in the experimental section. Each point represents the mean of two or three dishes, and the ranges were less than 10 % of the mean. Closed symbols are for albumin-containing incubation media and open symbols are for albumin-free media. ▵,□,○ represent Hep G2, 3T3-H.ras, and 3T3 respectively.

Table 3. Comparison of the efficiency of release of [^{3}H]PC from 3T3-H.ras with that from 3T3. [^{3}H]PC was isolated from the medium of pre-labelled cells at the indicated times as described in the Materials and Methods section. The radioactivity ratios represent the released labelled PC during a 1 hour chase interval divided by the amount of labelled cellular choline-lipids present at the beginning of the interval. Each value of the ratios is the mean ± S.D. of two or three analyses. C.C.-lipid means total cellular choline lipid

Incubation Time	medium PC/C.C.-lipid in 3T3-H.ras (%)	medium PC/C.C.-lipid in 3T3 (%)
1	0.19±0.01	0.30±0.05
2	0.19±0.02	0.32±0.04

Table 4. Comparison of the efficiency of release of [^{3}H]PC from Hep G2 with that from a normal rat hepatocytes. For the Hep-G2, [^{3}H]PC was isolated from the medium of pre-labelled cells at the indicated times as described in the Materials and Methods section. The radioactivity ratios represent the released labelled PC during a 1 hour chase interval divided by the amount of labelled cellular choline-lipids present at the beginning of the interval. Each value of the ratios is the mean ± S.D. of two or three analyses. C.C.-lipid means total cellular choline lipid

Incubation Time	medium PC/C.C.-lipid in Hep G2 (%)	medium PC/C.C.-lipid$^+$ in rat hepatocytes (%)
1	0.35±0.05	1.33±0.13
2	0.38±0.01	1.07±0.11

+; These values were calculated from the published data (35).

secrete PC 2.8-3.8 times more effectively than Hep G2 whereas 3T3 cells secrete PC 1.6 times more effectively than 3T3-H.ras.

Lysophosphatidylcholine and phosphatidylcholine secretion pools.

Comparison of the specific radioactivity of the cellular and secreted PC and LPC were measured by separation of the two lipids and quantitation of their ^{3}H radioactivities and phosphate contents. In the case of Hep G2 and 3T3-H.ras cells, the amount of LPC secreted into the medium was too small to allow phosphate measurement. I was only able to measure the radioactivity of secreted LPC and PC, which are shown in Figures 10 and 13. However, in the case of 3T3 cells, the phosphate content of PC and LPC secreted into the medium were measurable. Tables 5 and 6 show specific radioactivities of cellular and medium PC and LPC respectively. As we can see from the tables, the specific radioactivity of cellular PC was approximately the same as that of secreted PC. However, the specific radioactivity of LPC secreted into the medium was markedly lower than that of cellular LPC or of cellular PC. This LPC specific radioactivity also shows a steady increase during the incubation time while the

specific radioactivity of cellular PC shows little variation.

Table 5. Specific radioactivity of medium and cellular [^{3}H]PC of 3T3 cells. PC was isolated from prelabelled 3T3 cells and from the incubation media with albumin at the times indicated. Specific radioactivities of medium and cellular phosphatidylcholine were determined from radioactivity incorporated and phosphate amount of phosphatidylcholine as described under "Material and Methods". Each value of specific radioactivity is the mean ± S.D. of two analyses.

Time(h)	Cellular PC (dpm/nmol)	Medium PC (dpm/nmol)
0	$1.01(\pm0.13)\times10^4$	
1	$1.39(\pm0.09)\times10^4$	$1.06(\pm0.63)\times10^4$
2	$1.52(\pm0.04)\times10^4$	$1.13(\pm0.26)\times10^4$
4	$1.44(\pm0.06)\times10^4$	$1.34(\pm0.15)\times10^4$

Table 6. Specific radioactivity of medium and cellular [^{3}H]LPC of 3T3 cells. LPC was isolated from prelabelled 3T3 cells and from the incubation media with albumin at the times indicated. Specific radioactivities of medium and cellular lysophosphatidylcholine were determined from radioactivity incorporated and phosphate amount of lysophosphatidylcholine as described under "Material and Methods". Each value of specific radioactivity is the mean ± S.D. of two analyses.

Time(h)	Cellular LPC (dpm/nmol)	Medium LPC (dpm/nmol)
0	$9.22(\pm0.17)\times10^3$	
1	$1.20(\pm0.14)\times10^4$	$5.28(\pm0.41)\times10^3$
2	$1.36(\pm0.15)\times10^4$	$7.58(\pm0.09)\times10^3$
4	$1.31(\pm0.13)\times10^4$	$8.41(\pm1.28)\times10^3$

IV. Discussion

The 3T3 cell line established by G. Todaro and H. Green in 1962 from disaggregated Swiss mouse embryos has sensitivity to contact inhibition of cell proliferation (53). The 3T3-H.ras is a Harvey Sarcoma virus transformed nonproducer cell line. These cells have altered properties in vitro and lack contact inhibition of cell division (56). Hep G2, a liver cell line derived from a human hepatoblastoma, has been found to express a wide variety of liver-specific lipid metabolic reactions (40) and has similar lipid composition with normal human hepatocytes (41).

In this research, we compared LPC release from 3T3 cells with that from 3T3-H.ras cells. In addition, LPC release from Hep G2 is compared with previously published results on the rat hepatocytes (24-28).

3T3-H.ras cells more actively incorporate [^{3}H]choline than 3T3 cells and Hep G2.

The amount of [^{3}H]choline taken up by the three cell lines during the labelling period is obtained from the sum of the ^{3}H content of the cellular lipid and water-soluble fractions.

Expressed as a percent of the [^{3}H]substrate provided, these values are 32.8, 65.3, and 51.3 for 3T3, 3T3-H.ras, and Hep G2 respectively. Generally, choline uptaken is much more efficient in the transformed cell lines. In addition, by comparing this amount of radioactivity present in the cells' lipids at the start of the chase period (figure 4), it is clear that the 3T3-H.ras cells also incorporate this absorbed substrate into lipid more efficiently. It is not unexpected that transformed cells might be more active in PC synthesis than untransformed cells since their growth rate would be higher.

During the chase period, the ^{3}H content of cellular PC and LPC increases in Hep G2 and 3T3 cells but is relatively constant in 3T3-H.ras. Since the cytidylyltransferase is the rate-limiting enzyme in mammalian PC synthesis, phosphocholine is the major metabolic pool in the pathway. my attempt to minimize the size of this pool by incubation in a choline- and methionine-free medium prior to labelling appears to have been successful for 3T3-H.ras cells but less for Hep G2 and 3T3. Such differences in the size of the endogenous phosphocholine pools among the three cell lines would explain the higher incorporation of label into PC by 3T3-H.ras and also the steadily increasing label in PC during the chase period in Hep G2 and 3T3.

More efficient release of [^{3}H]LPC and [^{3}H]PC by albumin from nontransformed cells than from transformed cells.

After Sekas et al reported that an isolated rat liver directly secreted LPC in the presence of albumin, LPC secretion from isolated rat hepatocytes by albumin was studied (31-38). All of the research showed that rat hepatocytes strongly secreted LPC into albumin-containing medium while there was no secretion of LPC in the absence of albumin. Curiously, Brindley et al reported that ovine hepatocytes do not display the capacity to release LPC (38). Obviously, in transformed cells, choline and unsaturated fatty acid are always in demand in order to maintain a rapid growth rate. Our results (figure 10) show that not only Hep G2, the transformed liver cell line, but also the two cell lines derived from fibroblasts, 3T3 and 3T3-H.ras, release LPC into the medium in the presence of albumin but no LPC secretion occurs in its absence. It has been reasonably argued that physiologically the extra-essential fatty acid (unsaturated fatty acid) and choline in the untransformed cells may be transported to other tissues which need the fatty acid and choline.

A comparison of the efficiency with which [^{3}H]LPC is

released from the three cell lines is shown in Table 1. The amount of radioactive LPC appearing in the medium during a 1 hour time period as a percentage of the radioactive choline lipid in the cells at the beginning of the time period is a measure of the efficiency of release of this newly synthesized lipid. Clearly, the normal cell line, 3T3, is much more efficient compared with the transformed cells. Comparison of the Hep G2 data with that calculated from previously published work on the rat hepatocyte is shown in Table 2. Again the normal cell line releases [^{3}H]LPC more readily than does the transformed cell. Though Hep G2 is a human liver cancer cell and is different from the rat liver cell, it may give an insight into this process in a tumorous rat hepatocyte because PC metabolism of human and rat hepatocytes are similar (1). Since I was unable to measure the phosphate content of the secreted LPC for the two transformed cells, we are not able to measure these secretion efficiencies in terms of nmoles of LPC relative to nmoles of cellular choline lipid. Nevertheless, the differences between normal and transformed cells with respect to the release of newly synthesized LPC are significantly large.

The mechanism for LPC secretion is not well understood. It is generated by phospholipase A activities which have been found in almost every mammalian cell (16). In rat hepatocytes,

phospholipases A_1 and A_2 are found in the plasma membrane, microsomes, Golgi membrane, mitochondria, lysosomes, and cytosol (16). Many researchers in this field have suggested that LPC arises mainly by the action of phospholipase A_1 on PC because of the unsaturated nature of the secreted LPC (31, 32, 34-38). A major site for the origin of the LPC may be the plasma membrane, since lysophospholipase, the LPC-deacylating activity, does not occur in the plasma membrane (16). LPC so formed may then be sequestered into the medium by attachment to albumin or be reacylated to PC.

Albumin is the most abundant protein in human plasma, constituting about 50 percent (42±3.5 g/L) of the plasma proteins in the human. The protein exists as a monomer with a molecular weight of 66,200 and specifically binds a wide variety of biological materials such as LPC, PC,fatty acid, and some cationic and anionic ligands. It has been reported that the stoichiometry of binding is one molecule of LPC to one albumin molecule with an association constant, $K_A=4.3x10^4$ (55). Furthermore, the binding site for LPC is different from that for fatty acid.

The basis for the more efficient release of newly formed LPC from normal cells compared with transformed cells is not clear. As Brindley et al pointed out, this LPC, which contains

mostly unsaturated fatty acids, would supply other organs with choline and unsaturated fatty acids (34). We may assume that the tumor cell, in order to maintain rapid growth, might restrict the loss of materials such as choline and unsaturated fatty acids, which would be essential for new membrane synthesis. However, it is not clear why 3T3, and particularly 3T3-H.ras cells release PC into the medium even in the absence of albumin (figure 11) unless this PC is a component of a lipoprotein. The fibroblast, being an undifferentiated cell, may possess an active lipoprotein secretion pathway. The release of PC by Hep G2 is less surprising since Hep G2 is an hepatocyte cell line whose function it is to secrete lipoproteins.

With regard to the cell surfaces of the three cell lines, it is likely that the topography of the cell surface plays a role in the sequestering of LPC by albumin. When 3T3 cells are transformed by oncogenic viruses or chemical carcinogens, the transformed cells have an altered and simpler oligosaccharide moiety of their complex ganglioside, an acidic glycolipid (57). It has also been observed that the oligosaccharide moieties of tumor cell glycoproteins are different and simpler from those found in normal cell plasma membranes (51). Thus, the association of albumin with the cell surface may be affected in such a way that

there is a less effective binding of LPC from the transformed cell.

PC is secreted by the three cell lines regardless of the presence of albumin (figure 11). In Hep G2 (40) and rat hepatocytes (33,34), the PC secretion was reported as a normal function of the cell in which PC is secreted as one component of lipoprotein. The PC secretion from 3T3 cells may also be a lipoprotein component but that remains to be established. More than one explanation can be offered for the stimulation of PC secretion by albumin in the three cell lines. The most likely is that it results from the binding to albumin of PC in the plasma membrane. For this to occur, a tight association of albumin with the membrane would be needed. Differences in the effective association of albumin with the cell surface between transformed and normal cells may also account for the less efficient release of newly formed PC from the transformed cells (table 3 and 4). Thus, both LPC and PC release may be governed by the same mechanism. If the PC that appears in the medium has a lipoprotein source as is the case for Hep G2, it is conceivable that there may be an enhanced lipoprotein secretion in response to albumin. In the case of rat hepatocytes the stimulation of PC release by albumin is only slight, even less than that shown by Hep G2 (35). This might be anticipated if both sets of hepatocytes

are operating at or near maximum capacity for lipoprotein secretion under albumin-free conditions.

A discrete pool of PC is selected for the synthesis and release of LPC in 3T3 cells.

The specific radioactivity of cellular LPC in 3T3 cells was lower than the specific radioactivity of cellular PC as shown in Tables 5 and 6. Since the pool size of LPC is always very small, it is clear that cellular LPC is generated from newly synthesized and endogenous PC. More surprising is the observation that the specific radioactivity of the medium LPC was about 40 % of that of the cellular LPC at 1 hour of incubation and only increased to 66 % after 4 hours. Clearly, the pools of newly synthesized PC and LPC are not the major sources for the LPC that is secreted and this secretory pool only slowly equilibrates with the LPC generated from cellular PC. Brindley et al reached the same conclusion concerning separate pools in LPC production from their observation that LPC secreted from the female rat hepatocyte has a low content of arachidonic acid despite a relatively higher proportion of this acid in the cellular PC (38).

These observations are in sharp contrast to those made with cellular and secreted PC described below.

The specific radioactivity of PC made from [^{3}H]choline was approximately equal in 3T3 cells and the medium as shown in Table 4. This is not surprising considering the data reported from several studies on the rapid rate of equilibration of PC among microsomal, Golgi, and plasma membranes of rat liver (58). Other studies have shown a $t_{1/2}$= 2 minute at 25 °C for transfer of PC from the endoplasmic reticulum (the site of synthesis of PC) to the plasma membrane in Chinese hamster ovary cells (59). It is clear that the pathways by which PC and LPC appear in the medium originate from distinctly different pools of cellular PC. In support of the existence of different pools of cellular PC, Vance et al have found that in the case of lipoprotein secretion by cultured rat hepatocytes that there is a preference for PC made from choline rather than ethanolamine (50). In addition, from PC and phospholipid secretion using ^{3}H-serine, they concluded that there is no random and homogeneous labelling of phospholipid pools from radioactive precursors.

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